Ablation of calcitonin/calcitonin gene-related peptide-α impairs fetal magnesium but not calcium homeostasis

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McDonald, Kirsten R., Neva J. Fudge, Janine P. Woodrow, James K. Friel, Ana O. Hoff, Robert F. Gagel, and Christopher S. Kovacs. Ablation of calcitonin/calcitonin gene-related peptide-α impairs fetal calcium but not magnesium homeostasis. Am J Physiol Endocrinol Metab 287: E218–E226, 2004. First published March 23, 2004; 10.1152/ajpendo.00023.2004.—We used the calcitonin/calcitonin gene-related peptide (CGRP)-α gene knockout model (Ct/Cgrp null) to determine whether calcitonin and CGRPα are required for normal fetal mineral homeostasis and placental calcium transfer. Heterozygous (Ct/Cgrp+/−) and Ct/Cgrp null females were mated to Ct/Cgrp+/− males. One or two days before term, blood was collected from mothers and fetuses and analyzed for ionized Ca, Mg, P, parathyroid hormone (PTH), and calcitonin. Amniotic fluid was collected for Ca, Mg, and P. To quantify skeletal mineral content, fetuses were reduced to ash, dissolved in nitric acid, and analyzed by atomic absorption spectroscopy for total Ca and Mg. Placental transfer of 45Ca at 5 min was assessed. Ct/Cgrp null mothers had significantly fewer viable fetuses in utero compared with Ct/Cgrp+/− and wild-type mothers. Fetal serum Ca, P, and PTH did not differ by genotype, but serum Mg was significantly reduced in null fetuses. Placental transfer of 45Ca at 5 min was normal. The calcium content of the fetal skeleton was normal; however, total Mg content was reduced in Ct/Cgrp null skeletons obtained from Ct/Cgrp null mothers. In summary, maternal absence of calcitonin and CGRPα reduced the number of viable fetuses. Fetal absence of calcitonin and CGRPα selectively reduced serum and skeletal magnesium content but did not alter ionized calcium, placental calcium transfer, and skeletal calcium content. These findings indicate that calcitonin and CGRPα are not needed for normal fetal calcium metabolism but may regulate aspects of fetal Mg metabolism.

FETAL MINERAL HOMEOSTASIS is regulated differently from adult homeostasis, reflecting the unique needs of the developing fetus, which include maintaining serum mineral concentrations higher than in the mother, actively transporting mineral across the placenta, and mineralizing the developing skeleton (23, 27). Parathyroid hormone (PTH) and PTH-related protein (PTHRP) have been shown to play interlocking roles in the regulation of fetal blood calcium, placental calcium transfer, and formation of the fetal skeleton (20, 24, 28, 29). Vitamin D, calcitriol, and its receptor have been shown in a number of experimental models to be relatively unimportant for fetal mineral homeostasis (see review in Ref. 23), unlike in the adult, where these elements are critically required.

The role of calcitonin in fetal mineral metabolism has not been extensively examined. Calcitonin is expressed by human thyroid C cells early in gestation (33), and it circulates in the fetal circulation at levels that are higher than in the mother (23, 27). With respect to serum calcium regulation, Garel and colleagues established that pharmacological administration of calcitonin (16) or of calcitonin antiserum (15) altered the serum calcium of fetal rats in predictable and opposite directions. However, Care et al. (9) found that fetal thyroidecotomy with subsequent thyroxine replacement did not affect the fetal blood calcium in sheep, indicating that the physiological amounts of calcitonin derived from the fetal thyroid may not be required to maintain a normal serum calcium.

With respect to placental calcium transfer, the potential role of calcitonin was indirectly examined by Barlet and colleagues in two reports. Skeletal mineral content was reduced in thyroidecotomized fetal sheep that received thyroxine but not calcitonin supplementation (4), and pharmacological doses of calcitonin decreased PTHrP-stimulated increases in fetal skeletal calcium content (5). However, placental calcium transfer was not directly measured in those two studies; instead, skeletal ash weight and mineral content were measured several days after initiation of treatment. The sole study that directly measured placental calcium transfer was that of Care et al. (9), in which fetal thyroidecotomy with subsequent thyroxine replacement did not alter placental calcium transfer in fetal sheep as determined in a placental perfusion model.

The previously cited studies did not adequately examine the physiological role of calcitonin in fetal mineral homeostasis, because pharmacological manipulations were done, and because the surgical removal of the thyroid did not create a completely calcitonin-deficient state. It has since been established that calcitonin is also produced in the central nervous system, late pregnant and lactating breast, uterus, and placenta (3, 8, 25). In particular, the presence of calcitonin and its receptor in the placenta of humans and mice indicates that it may play a role in some aspect of placental function (25).

To examine a model that is completely deficient in physiological amounts of calcitonin, we have utilized the calcitonin/calcitonin gene-related peptide-α (Ct/Cgrp) gene knockout model. CGRPα is an alternative splice product of the calcitonin gene; a second gene produces CGRPβ. Therefore, Ct/Cgrp null...
mice completely lack calcitonin but still produce CGRP through the CGRPbeta gene. Ablation of Ct/Cgrp in mice has been shown to result in a phenotype characterized by increased bone mass, increased bone formation, and preservation of bone mass during acute estrogen withdrawal from ovarioectomy (18), but its role in fetal mineral homeostasis had not yet been reported.

In this study, we tested the hypothesis that calcitonin is not required for the regulation of the fetal blood calcium and normal skeletal development and mineralization in uterus. We utilized the Ct/Cgrp null mice as a model for absence of calcitonin, and we contrasted the phenotype of these fetal mice with that of their wild-type (wt) and heterozygous (Ct/Cgrp) and CGRP. The nature of the model meant that we tested for the effects of absence of both products of Ct/Cgrp, calcitonin and CGRPalpha. We found that absence of calcitonin and CGRPalpha results in modest abnormalities of magnesium metabolism but no alteration in calcium metabolism.

MATERIALS AND METHODS

Animal husbandry. Ct/Cgrp gene knockout mice were obtained by targeted disruption of the murine gene in embryonic stem cells, as previously described (18). The original strain was backcrossed into Black Swiss (Taconic, Germantown, NY) for at least four generations, and the colony was maintained through breeding heterozygous mice together. Ct/Cgrp+/- males and females were mated to create pregnancies in which wt, Ct/Cgrp+/-, and Ct/Cgrp null fetuses were present. Ct/Cgrp+/- males and Ct/Cgrp null females were also mated to yield pregnancies with Ct/Cgrp+/- and Ct/Cgrp null fetuses. The Ct/Cgrp+/- and Ct/Cgrp null females were first-degree relatives of each other. Mice were mated overnight; the presence of a vaginal mucus plug on the morning after mating marked gestational day 0.5. Normal gestation in these mice is 19 days. All mice were given a standard chow (1% calcium) diet and water ad libitum. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Genomic DNA was obtained from fetal tails, and genotyping was accomplished by PCR with three primers that were specific to the Ct/Cgrp gene sequence and the neomycin cassette, in a single-tube, 36-cycle PCR reaction utilizing a PTC-200 Peltier Thermal Cycler (MJ Research, Cambridge, MA). These primers utilized the following specific sequences: CAG GAT CAA GAG TCA CCG CT (CT-1), GGA GCC TGC GCT CCA GCG AA (CT-3), and GGT GGA TGT GGA ATG TGT GC (CT-N).

At the time of each cesarian section (day 17.5 or 18.5 of gestation), the number of viable fetuses present was counted. In separate studies, the number of live pups present at the time of weaning was counted to determine the postnatal litter size. This litter number is more variable than the number of fetuses, due to normal loss of pups in the neonatal period from factors such as maternal culling of the litter. 

Chemical and hormone assays. Whole blood, serum, and amniotic fluid were collected using methods previously described (29). Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca++/pH Analyzer (Chiron Diagnostics, East Walpole, MA). SerumPTH was measured on embryonic day 18.5 (ED 18.5) fetuses using a rodent PTH-(1–34) ELISA kit (Immutopics, San Clemente, CA); the stated detection limit of the assay was 1.6 pg/ml. Serum calcitonin was measured using an immunoradiometric assay (IRMA; Immunoetics) developed to rat calcitonin, with sera from three or four fetuses pooled together to obtain sample volumes of 100 µl. These pooled samples were then diluted with zero standard to meet the sample size requirements of the assay. No pooling was required for maternal samples. Total calcium, magnesium, and phosphorus were measured using photometric assays (Sigma-Aldrich, Oakville, ON).

Placental calcium transfer. This procedure has been described in detail elsewhere (28). Briefly, pregnant dams on ED 17.5 were given an intracardiac injection of 50 µCi 45Ca and 50 µCi 51Cr-EDTA. Five minutes later, the dams were killed, and each fetus was removed. The radioactivity ratio of 45Ca to 51Cr was determined for each fetus with a γ-counter and a liquid scintillation counter, respectively. The data were normalized to the mean 45Ca/51Cr activity ratio of the Ct/Cgrp+/- fetuses in each litter so that the results from different litters could be compared.

Tissue collection. For in situ hybridization and immunohistochemistry of fetal bones, whole fetuses (ED 17.5 or 18.5) were placed in 10% formalin after the first incision in the abdomen to prevent its gaseous expansion. For in situ hybridization of placentas, the maternal circulation was first perfused with phosphate-buffered saline followed by paraformaldehyde, after which the placentas were removed from the uterus and placed into fixative (29). After 12–24 h in the fixative, the limbs and placentas were removed and separately processed, embedded in paraffin, and cut into 5-µm sections.

Fetal ash and skeletal mineral assays. With methods previously described (24), intact fetuses (ED 18.5) were reduced to ash in a furnace (500°C × 24 h), and the ash was assayed for calcium and magnesium on a Perkin Elmer 2380 Atomic Absorption Flame Spectrophotometer. Because fetal size varied from litter to litter and would affect the individual measurements (large litter, smaller fetuses; small litter, larger fetuses), the data were normalized to the mean value of the heterozygotes within each litter. The heterozygotes were chosen as the baseline for this comparison because, on average, they accounted for 50% of the fetuses in a given litter.

Riboprobe and DNA probe labeling. For in situ hybridization, the plasmids were linearized with appropriate restriction enzymes and labeled with 125 µCi of 35S-UTP by use of an SP6/T7 Transcription Kit (Promega/Fisher Scientific, Burlington, ON) and the appropriate polymerase. Unincorporated nucleotides were removed with the NucTrap columns (Strategene, La Jolla, CA).

Growth plate- and bone-related cDNAs included pro-alpha1(I) chain of human type I collagen (6), pro-alpha1(II) chain of rat type II collagen (22), H4 histone (39), and mouse type X collagen (1) (gift of K. Lee); mouse osteocalcin (14), rat osteopontin (36), rat alkaline phosphatase (40), and murine cartilage matrix protein (matrilin) 1 (2) (gifts of B. Lanske); rat PTHrP (21) (gift of H. M. Kronenberg); murine interstitial collagenase (17) and murine 92-kilodalton gelatinase (type IV collagenase or MMP-9) (38) (gifts of S. M. Krane). Placental cDNAs used included murine calbindin-D(32S) (35) (gift of S. Christakos), human Ca2+-ATPase (30) (gift of R. Kumar), murine alpha-fetoprotein (gift of Margaret Baron), murine placental lactogen (19) and murine prolactin (34) (gifts of D. Linzer), and murine nodin (41) (gift of M. Kuehn).

In situ hybridization. In situ hybridization was performed on 5-µm tissue sections, as described previously (32). Hybridization was performed in a humidified chamber (16 h, 55°C) with the labeled riboprobe diluted 1:20 in the hybridization solution. Sections were successively washed, RNAse treated, and dehydrated in graded ethanol (EthOH) series. An overnight exposure of the slides to plain X-ray film enabled an estimate of exposure time for the liquid emulsion step. Slides were then dipped into NTB-2 liquid emulsion, dried, stored in light-tight boxes, and kept at 4°C until developed (2–6 wk). The emulsion was developed using standard developer and fixer, and the sections were counterstained with hematoxylin-eosin.

All comparisons of wt with Ct/Cgrp null animals were made between tissues obtained from within the same litter, and which had been processed, embedded, and sectioned at the same time. All comparative sections were always hybridized together with the same probe and washed together to validate the comparison and to minimize artifacts. Assessments of signal intensity were determined in a blinded fashion (no knowledge of the genotype). The reproducibility of the results was confirmed independently on at least three separate litters of each knockout colony.
Histology. Sections (5-μm) were deparaffinized, rehydrated in a graded EtOH series, and transferred to distilled water. For morphological assessment of the growth plate, sections were stained with hematoxylin and eosin, or 1% methyl green, and then dehydrated and mounted. For von Kossa staining, the sections were transferred to 1% aqueous silver nitrate solution and exposed for 45 min under a strong light. They were then thrice washed in distilled water, placed in 2.5% sodium thiosulfate (5 min), and thrice washed again in distilled water. Finally, they were counterstained with methyl green, dehydrated in 1-butanol and xylene, and mounted.

Alican red S and Alican blue preparations. Fresh fetuses (ED 18.5) were obtained and the skin, viscer.a, and adipose tissue were carefully removed. In individual scintillation vials, the fetuses were fixed in 95% EtOH for 5 days, followed by acetone for 2 days to remove the remaining fat and firm up the specimen. After this, the fetuses were stained for 3 days in 10 ml of freshly prepared staining solution at 37°C (1 volume 0.3% Alican blue 8GS in 70% EtOH-1 volume 0.1% Alizarin red S in 95% EtOH-1 volume acetic acid-17 volumes 70% EtOH). They were then washed in distilled water and immersed in 1% aqueous KOH until the fetal skeleton was clearly visible through the surrounding tissue (~12–48 h). They were cleared in 1% KOH containing increasing concentrations (20, 50, and 80%) of glycerin (7–10 days at each step). Finally, they were transferred into 100% glycerin for permanent storage.

Statistical analysis. Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, Evanston, IL). ANOVA was used for the initial analysis; Tukey’s test was used to determine which pairs of means differed significantly from each other. Two-tailed probabilities are reported, and all data are presented as means ± SE.

RESULTS

Maternal effects of calcitonin/CGRPα deletion. Possible maternal effects of calcitonin/CGRPα deletion were examined by comparing pregnant and nonpregnant Ct/Cgrp+/− and Ct/Cgrp null mothers. Ct/Cgrp+/− and Ct/Cgrp null mothers mated readily and conceived with the same frequency, but the number of viable fetuses in utero of Ct/Cgrp null females was significantly lower than the number of Ct/Cgrp+/− females when assessed on ED 17.5 or 18.5 at C section (Fig. 1A). Resorption sacs were infrequent and no different in number between litters from Ct/Cgrp+/− and Ct/Cgrp null mothers, indicating that the difference in fetal number developed before the embryonic stage. In contrast, although the trend to lower numbers persisted after birth, the difference was not statistically significant by the time of weaning (Fig. 1B).

On the day before expected birth, maternal ionized calcium was 1.28 ± 0.02 mmol/l in Ct/Cgrp+/− and 1.29 ± 0.02 mmol/l in Ct/Cgrp+/− null dams (P = not significant). These values were not different from the wt or corresponding nonpregnant values (1.28 ± 0.02 mmol/l in Ct/Cgrp+/− and 1.30 ± 0.02 mmol/l in Ct/Cgrp+/− null), indicating that maternal absence of Ct/Cgrp did not impair the mother’s ability to maintain her calcium concentration during the time frame when calcium transfer across the placenta is at its peak. In contrast to the stability of calcium across genotypes and during pregnancy, maternal magnesium was reduced during pregnancy in Ct/Cgrp null mothers. Maternal serum magnesium was 1.17 ± 0.08 mmol/l in nonpregnant Ct/Cgrp null mothers and was reduced to 0.98 ± 0.07 mmol/l in pregnant Ct/Cgrp null mothers on the day before expected birth (P < 0.001). In contrast, serum magnesium was not different between the pregnant and nonpregnant state in Cgrp+/− mothers (1.17 ± 0.05 and 1.14 ± 0.04 mmol/l, respectively).

Placental calcium transfer. To definitively determine whether calcitonin and CGRPα are required by the fetus to maintain placental calcium transfer, we measured the amount of 45Ca transferred to each fetus within 5 min (normalized by 51Cr). In Ct/Cgrp null fetuses obtained from Ct/Cgrp+/− mothers, placental calcium transfer occurred at a rate that was indistinguishable from that of Ct/Cgrp+/− and wt siblings (Fig. 2A). We further assessed placental calcium transfer in fetuses of Ct/Cgrp null mothers to determine whether maternal absence of calcitonin and CGRPα, or the combined absence of maternal and fetal calcitonin and CGRPα, would alter the rate of placental calcium transfer. No difference in the relative rate of 45Ca transfer was noted between Ct/Cgrp+/− and Ct/Cgrp null fetuses of Ct/Cgrp null mothers (Fig. 2B), and the transfer rate was the same as in fetuses of Ct/Cgrp+/− mothers. Thus placental calcium transfer was normal regardless of whether the fetus, the mother, or both lacked calcitonin and CGRPα.

Serum and amniotic fluid mineral physiology. Fetal lack of calcitonin and CGRPα did not alter fetal regulation of the blood calcium, as indicated by the normal ionized calcium and fetal-maternal calcium gradient in wt, Ct/Cgrp+/−, and Ct/Cgrp null fetuses obtained from Ct/Cgrp+/− dams (Fig. 3A). Furthermore, fetal blood calcium regulation was also unaffected by maternal absence of calcitonin and CGRPα, or combined maternal and fetal absence of calcitonin and CGRPα, because Ct/Cgrp+/− and Ct/Cgrp null fetuses had the same ionized calcium level regardless of whether the mother was Ct/Cgrp+/− or Ct/Cgrp null (Fig. 3A and B). In contrast to the normal ionized calcium levels, serum magnesium concentrations were significantly reduced in Ct/Cgrp null fetuses both when the mother was Ct/Cgrp+/− (Fig. 3C) and when she was Ct/Cgrp null (Fig. 3D). Serum phosphorous levels trended lower in Ct/Cgrp null fetuses but were not significantly different from those of their littermates in litters obtained from Ct/Cgrp+/− and Ct/Cgrp null dams (Fig. 3E and F).

In contrast to the altered serum magnesium concentrations in Ct/Cgrp null fetuses, there were no significant differences in the concentrations of magnesium, calcium, or phosphorus in amniotic fluid (Table 1).
Serum PTH was not significantly different among the genotypes, but the mean values trended slightly higher from wt to null (Fig. 4).

Serum calcitonin was measured in fetuses obtained from both Ct/Cgrp+/− and Ct/Cgrp null mothers. Within offspring obtained from Ct/Cgrp+/− mothers, calcitonin dropped in a step-wise fashion from wt to Ct/Cgrp+/− to Ct/Cgrp null (undetectable) (Fig. 5A). Similar results were obtained in fetuses from Ct/Cgrp null mothers, confirming that absence of maternal calcitonin did not affect the fetal calcitonin level (Fig. 5B). Furthermore, fetal calcitonin was undetectable in null fetuses obtained from both Ct/Cgrp+/− and Ct/Cgrp null mothers, confirming that calcitonin cannot cross the placenta from mother to fetus in appreciable amounts. The serum calcitonin level was also undetectable in Ct/Cgrp null mothers, confirming that the gene deletion had eliminated calcitonin. Although the stated detection limit of the assay is 2 pg/ml in rat serum, it is likely that the level of −8 pg/ml represents a true zero for this assay in mouse serum under these experimental conditions.

Skeletal morphology and mineral content. The fetal skeleton is intimately involved in calcium metabolism, and it mineralizes during late gestation. We have previously demonstrated that it is possible for placental calcium transfer to be normal but the skeleton not accrete mineral at a normal rate (24). Therefore, the fetal skeleton was examined to determine whether lack of calcitonin and CGRP altered skeletal development, skeletal mineral content, or gene expression within the growth plate. Furthermore, the skeletons were examined to determine whether the increased bone mass observed in adult Ct/Cgrp null mice (18) could be detected during fetal development.

The gross morphology and mineralization of the fetal skeletons were examined using intact but cleared fetuses that had

| Table 1. Amniotic fluid mineral concentrations |
|---------------|---------------|---------------|
|               | Calcium       | Magnesium     | Phosphorus    |
| wt            | 1.73±0.23 (16) | 1.15±0.09 (12) | 2.3±0.2 (12)  |
| Ct/Cgrp+/−    | 1.27±0.17 (30) | 1.13±0.06 (23) | 1.9±0.2 (20)  |
| Ct/Cgrp null  | 1.29±0.21 (15) | 1.13±0.07 (15) | 2.2±0.2 (13)  |

Values are means ± SE expressed in mmol/l. wt, Wild-type; Ct/Cgrp+/−, heterozygous calcitonin/calcitonin gene-related peptide (CGRP)-α-; Ct/Cgrp null, CGRPα knockout animals. Nos. of fetuses are indicated in parentheses.
been stained with Alcian blue (for cartilage) and Alizarin red S (for mineralized bone). The fetal skeletons exhibited no detectable abnormalities, including normal length and morphology of the long bones in the appendicular skeleton (Fig. 6, a and b). The relative distribution of mineral also appeared to be normal.

The mineral content of fetal skeletons was determined by obtaining the ash weights of fetuses (which represent largely mineral), and then assaying the ash to determine the calcium and magnesium content. No differences were found in ash weights of fetuses of all of the genotypes, whether obtained from Ct/Cgrp+/− or Ct/Cgrp null mothers (Table 2). This finding confirms the qualitative finding (from skeletal preparations stained with Alizarin red S) that the skeletal mineral content was unaltered by absence of calcitonin and CGRPα. The ash was further analyzed by atomic absorption spectroscopy to determine its calcium and magnesium content (Table 2). Results were calculated in milligrams of calcium or magnesium per gram of ash and were normalized to the mean heterozygote value for each litter. The calcium content of the fetal skeletons did not differ among the genotypes, whether obtained from Ct/Cgrp+/− or Ct/Cgrp null mothers (Table 2). This finding confirms the qualitative finding (from skeletal preparations stained with Alizarin red S) that the skeletal mineral content was unaltered by absence of calcitonin and CGRPα. The ash was further analyzed by atomic absorption spectroscopy to determine its calcium and magnesium content (Table 2).

Growth plate morphology and gene expression. The growth plates were examined histologically, and the distribution of mineral within the long bones was examined semiquantitatively using the von Kossa method. In this method, silver displaces calcium to create black silver phosphate and silver carbonate complexes; because calcium is the only known cation that binds to these insoluble anions in organic tissue, the method is considered to be sufficiently specific for calcium (7, 37). With this method, the mineral present in Ct/Cgrp null growth plates appeared to be distributed normally compared with wt siblings (Fig. 6, c and d). In addition, endochondral bone formation appeared to be normal, as evidenced by the length of the growth plate, chondrocyte morphology from the proliferative to the hypertrophic zones, peristeal thickness, and the lengths of the long bones.

The growth plates and long bones were further examined by in situ hybridization to determine whether the expression of

Fig. 4. Serum parathyroid hormone (PTH) in fetuses obtained from Ct/Cgrp+/− mothers. Values are means ± SE. Nos. of fetuses are indicated in parentheses.

Discussion

In this study we measured several parameters of mineral and bone metabolism to determine whether Ct/Cgrp and its two products, calcitonin and CGRPα, are critically required for normal fetal-placental mineral homeostasis and fetal skeletal development.

Our data clearly show that lack of calcitonin and CGRPα resulted in a smaller number of viable fetuses by the day before expected birth, with no difference noted in the number of genes by chondrocytes, osteoblasts, and osteoclasts was altered by the absence of calcitonin and CGRPα. The distribution pattern and intensity of expression of the mRNAs for types I, II, and X collagens, H4 histone, cartilage matrix protein (matrilin 1), PTHrP, osteoblast markers (osteopontin, osteocalcin, interstitial collagenase, and alkaline phosphatase), and an osteoclast marker (92-kDa gelatinase or type IV collagenase or matrix metalloproteinase-9) were all examined. No differences were seen between wt and Ct/Cgrp null tibias and femurs (Fig. 6, e–p, and data not shown).

Placental morphology and gene expression. Finally, because calcitonin and its receptor are expressed within the placenta and have been thought to be important for normal placental function (25), we examined placentas obtained from wt and Ct/Cgrp null fetuses. Placentas were individually weighed, placental structure was examined microscopically, and the expression of specific placental genes was studied to determine whether placental structure or function was altered by the absence of calcitonin and CGRPα. Placental weight was unaltered among the fetal genotypes (not shown). Placental structure was normal, including a normal distribution of trophoblast cell types (labyrinthine, spongiotrophoblasts, and giant cell trophoblasts) and a normal amount of intraplacental yolk sac (Fig. 7, a, b, e, and f). No difference was noted in the intensity of expression of the mRNAs for calbindin-D28k or Ca2+-ATPase, both known to be required for the placental transfer of calcium (Fig. 7, c, d, g, and h). In addition, the relative expression of the trophoblast markers placental lactogen, nodal, and prolifemin and of the extraplacental yolk sac marker α-fetoprotein was unaltered by loss of calcitonin and CGRPα (data not shown).

Discussion

In this study we measured several parameters of mineral and bone metabolism to determine whether Ct/Cgrp and its two products, calcitonin and CGRPα, are critically required for normal fetal-placental mineral homeostasis and fetal skeletal development.

Our data clearly show that lack of calcitonin and CGRPα resulted in a smaller number of viable fetuses by the day before expected birth, with no difference noted in the number of
nonviable or resorbed gestational sacs. It was maternal loss of calcitonin and CGRP that was associated with the reduction in the number of viable fetuses; in contrast, fetal loss of Ct/Cgrp did not affect the number of viable fetuses, because Ct/Cgrp null fetuses were present in the expected Mendelian ratios in both Ct/Cgrp/H11001/H11002 and Ct/Cgrp null uteri. These findings may have relevance to previous evidence that calcitonin is expressed in the endometrium during the time of implantation (31) and that calcitonin may be required for the blastocyst to implant (42). The decrease in fetal number appeared to persist into the neonatal period but was not statistically significant; maternal culling of the litter after delivery accounts in large part for why the postnatal litter size is more variable than the number of viable fetuses in vitro.

We noted no difference in skeletal ash weight or skeletal calcium content in fetal mice, indicating that lack of calcitonin and CGRPα in either the mother or the fetus did not impair the amount of calcium accreted by the skeleton or total bone mass at the end of gestation. Furthermore, we observed no abnormality in endochondral bone formation, including morphology and gene expression within the growth plates of the fetal long bones. These findings compare with the observation of Hoff et al. (18) that Ct/Cgrp null mice had a progressive increase in bone mass that became apparent between 1 and 3 mo of age (18), and the more recent finding of Dacquin et al. (12) that heterozygous ablation of the calcitonin receptor also leads to a higher bone mass. Our findings confirm that the increase in bone mass does not occur before birth in the Ct/Cgrp null animal.

Fig. 6. Skeletal morphology and gene expression. Skeletal morphology of wild-type (wt, a) and Ct/Cgrp null (b) fetal skeletons (ED 18.5) stained with Alizarin red S (mineral) and Alcian blue (cartilage). Crown-rump length, lengths of long bones, and mineralization pattern were unaltered in Ct/Cgrp null (b) compared with its wt sibling (a), von Kossa stains (c and d) of fetal growth plates counterstained with methyl green. Overall morphology and length of growth plate was normal in Ct/Cgrp null (b) compared with wt animals, and the amount of mineral (black) also did not differ between Ct/Cgrp null and wt animals. In situ hybridization studies (e–p) of fetal tibial growth plates (ED 18.5) in wt (e–j) and Ct/Cgrp null (k–p) siblings. Representative dark-field images are shown of collagen II (COL-II), collagen X (COL-X), collagen I (COL-I), osteopontin (OP), 92-kDa gelatinase (GEL), and osteocalcin (OC). No significant difference is seen between wt and Ct/Cgrp null growth plates. Scale bars, 0.5 cm (a and b) and 100 μm (in remaining panels).
Table 2. Ash weight, skeletal calcium content, and skeletal magnesium content normalized to mean value of Ct/Cgrp +/− fetuses within each litter

<table>
<thead>
<tr>
<th>Ct/Cgrp +/− Mothers</th>
<th>Ct/Cgrp Null Mothers</th>
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<td>Ash weight</td>
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<tr>
<td>Ct/Cgrp null</td>
<td>100.1 ± 2.5 (40)</td>
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Values are means ± SE. Nos. of fetuses are indicated in parentheses. *P < 0.025 vs. Ct/Cgrp +/−.

Although fetal and maternal serum calcium was unaltered by absence of calcitonin and CGRPα, we observed a step-wise decrease in fetal serum magnesium from wt to Ct/Cgrp null animals and a decrease in serum magnesium of pregnant Ct/Cgrp null mothers. Furthermore, although skeletal calcium content was unaffected by absence of calcitonin and CGRPα, skeletal magnesium content was reduced in Ct/Cgrp null fetuses of Ct/Cgrp +/− mothers. These findings suggest that absence of calcitonin or CGRPα (or of both) impairs magnesium homeostasis, including regulation of the magnesium concentration in the circulation of fetuses and pregnant mothers and the amount of magnesium that is accreted by the fetal skeleton. The mechanism by which calcitonin or CGRP affects magnesium homeostasis is unknown. PTH is unlikely to be a factor, because PTH and phosphorus were not significantly altered in Ct/Cgrp null fetuses.

Amniotic fluid mineral content is a surrogate measure of mineral excretion by the fetal kidneys, and in our hands, this measurement discriminates between high and low levels of calcium and magnesium in states in which the kidneys are expected to have high vs. low filtered loads of these minerals (26, 29). In the present studies, we found no significant difference among genotypes in the amniotic fluid content of calcium, magnesium, or phosphorus. Thus the fetal kidneys of the Ct/Cgrp null fetuses do not appear to be conserving magnesium in response to the low serum magnesium.

Our findings indicate definitively that calcitonin and CGRPα are not required to regulate fetal-placental calcium transfer, as confirmed by the lack of any alteration in the rate of placental 45Ca transfer at 5 min, the normal amount of calcium accreted by the fetal skeleton, the normal expression of Ca2+-ATPase and calbindin-D9k (both critical components of transcellular calcium transfer in trophoblasts and intraplacental yolk sac), and the normal structure and weight of the placenta. Furthermore, the additional absence of calcitonin and CGRPα in the mother had no discernible effect on the rate of placental calcium transfer. It was not technically possible to measure the placental transfer of magnesium because the isotope must be custom-made at formidable expense, and its short half-life would mean that more than two half-lives would pass during transport to our laboratory, rendering it useless. Thus it remains unknown whether placental magnesium transfer is altered in Ct/Cgrp null fetuses.

We have previously used Ct/Cgrp null placentas to confirm the presence of calcitonin mRNA and protein within the placenta, and we have also documented the presence of the calcitonin receptor in the placenta (25). The presence of both calcitonin and its receptor in placenta is consistent with a possible role for calcitonin in some aspect of placental function. Our findings do not indicate a role for calcitonin in placental calcium transfer, but they are compatible with a role for calcitonin in placental magnesium transfer.

The factors that regulate magnesium metabolism are not clearly defined in the adult and have not been explored at all in the fetus. There is only very limited evidence that calcitonin...
may contribute to the regulation of magnesium metabolism in the adult. A number of investigators have demonstrated that calcitonin stimulates the conservation of both magnesium and calcium by the renal tubules (10, 11, 13), but those observations may represent pharmacological and not physiological effects of calcitonin. We observed no alteration in the apparent renal excretion of calcium, magnesium, and phosphorus in Ct/Cgrp null fetuses.

In summary, we examined several indexes of mineral metabolism in this study of Ct/Cgrp ablation in fetal mice. We found that the serum magnesium level and skeletal magnesium content were reduced in Ct/Cgrp null fetuses but that parameters of calcium metabolism (serum calcium, placental calcium transfer, and skeletal calcium content) were unaltered. Fetal skeletal weight, growth plate morphology, and gene expression within the growth plates were normal in Ct/Cgrp null fetuses, in contrast to the increased bone mass that was observed in Ct/Cgrp null adults. Placental morphology, weight, and gene expression were also unaltered. Ct/Cgrp null mothers had pregnancies with significantly fewer viable fetuses, but this difference was not significant after maternal culling of the litters in the neonatal period. Taken as a whole, these results indicate that calcitonin and CGRPα are required for some determinant of gestational litter size (possibly implantation of the blastocyst) and for normal fetal magnesium homeostasis. Calcitonin and CGRPαs are not essential for later survival and development of the fetus, and they are not required to maintain placental calcium transfer, fetal calcium homeostasis, and calcium accretion by the fetal skeleton.

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